Discovery of metabolic resistance to neonicotinoids in green peach aphids (*Myzus persicae*) in Australia

Running title: Resistance in *Myzus persicae* to neonicotinoids in Australia

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Abstract

BACKGROUND: *Myzus persicae* is a serious pest that attacks a broad range of agricultural crops. This species has developed chemical resistance to many insecticides globally and within Australia resistance to multiple chemical groups has been identified. Resistance to neonicotinoid insecticides has been discovered in several countries, but has not previously been confirmed in Australia. We use biomolecular assays and bioassays on field-collected populations to investigate neonicotinoid resistance in *M. persicae* within Australia. RESULTS: Several geographically and genetically distinct populations showed evidence for resistance in bioassays. Genetic markers identified the mechanism of neonicotinoid resistance in Australia is metabolic resistance through the enhanced expression of a cytochrome P450 gene, *CYP6CY3*. CONCLUSIONS: *M. persicae* populations in parts of Australia are now resistant to four different insecticide chemical groups, raising concerns about the long-term management of this pest. While higher copy numbers of *CYP6CY3* were seen in all resistant populations, the number of gene copies was not strongly correlated with the level of resistance as determined by LD_{50} values generated through bioassays. This finding sheds further light on the complexity of the P450 genes at regulating neonicotinoid resistance.

Keywords: insecticide resistance, Australia, neonicotinoid, P450 gene, *Myzus persicae*

1. INTRODUCTION

The green peach aphid, *Myzus persicae* (Sulzer) is an important agricultural pest worldwide. *Myzus persicae* attacks plant hosts from over 40 different families, and can transmit more than 100 plant viruses.[1] In Australia, a variety of horticultural and broadacre crops are targeted by *M. persicae*, including canola, lupins, field peas, cruciferous vegetables, capsicums, eggplant and tomatoes.[2] They are also a sporadic pest of citrus, pome/stone fruits and cut flowers. Feeding damage from large *M. persicae* populations can affect crop establishment, stunt plant growth and reduce crop yields,[1, 3] however plant virus transmission by *M. persicae* is generally more economically important. In canola alone, viruses transmitted by *M. persicae* can reduce crop yields by up to 34%.[4] Furthermore, the secretion of honeydew by aphids can cause secondary fungal growth (i.e. sooty moulds), which inhibits photosynthesis and can further decrease plant growth. When deposited on fruit, honeydew and sooty mould greatly reduces the marketability of produce.

The principal control method for *M. persicae* in Australia is through insecticide applications[5, 6] and there are approximately 200 insecticide products registered to control *M. persicae* in Australia (APVMA (https://portal.apvma.gov.au/pubcris/)). Over-reliance on chemical control is problematic as, worldwide, *M. persicae* has developed resistance to 74 insecticides with different modes of action as classified by the Insecticide Resistance Action Committee (IRAC), including carbamates (1A), organophosphates (1B), organochlorines (2A), pyrethroids (3A) and neonicotinoids (4A) (ARPD (http://www.pesticideresistance.org/)). The most common insecticides used to target *M. persicae* in Australian crops are synthetic pyrethroids, organophosphates, carbamates, and neonicotinoids.[6] Recent field surveys of *M.*

persicae populations, coupled with resistance assays, has found widespread resistance to each of these chemical groups, with the exception of neonicotinoids.[6] The biochemical and molecular mechanisms of insecticide resistance in *M. persicae* have been extensively studied and their presence confirmed in Australia.[6] Enhanced expression of *E4/FE4* esterase confers resistance to organophosphates, (mono-methyl) carbamates and, to a lesser extent, pyrethroids by sequestering and possibly metabolising these insecticides before they reach the nervous system. Mutation (S431F) of the acetylcholinesterase enzyme results in resistance to dimethyl carbamates. Several mutations (L1014F, M918T, M918L) in the voltage-gated sodium channel individually and/or in combination confer resistance to pyrethroids.[7, 8]

Although several other chemical groups are registered for *M. persicae* control in some crops (e.g. tetramic acids, sulfoximines), neonicotinoids are often considered the 'final frontier' of insecticide control for *M. persicae* within Australia. As well as being registered widely as a foliar spray, neonicotinoids are commonly used in grain crops as a seed coating prior to sowing and are applied through drip irrigation lines in many vegetable crops. Neonicotinoids such as thiamethoxam, clothianidin and acetamiprid are mostly unaffected by the mechanisms conferring resistance to organophosphates, carbamates and pyrethroids, although imidacloprid has been shown to be partially affected by enhanced expression of *E4/FE4* esterase.[9] However, specific resistance mechanisms to neonicotinoids in *M. persicae* have been identified worldwide. Resistance was first reported in Greece in 2007,[9, 10] and over the last 9 years, has emerged in other European countries and the United States (ARPD (http://www.pesticideresistance.org/)). The most common neonicotinoid resistance mechanism in *M. persicae* is through amplification and enhanced expression of the

P450 gene *CYP6CY3*.[11] A second mechanism has recently been discovered that confers significantly higher levels of resistance via a point mutation (R81T) in the loop D region of the ß1 subunit of nicotinic acetylcholine receptor.[12] This mechanism was identified following the collection of aphids from France in 2009 that survived field applications of neonicotinoids. This resistance mechanism has since been detected in holocyclic populations across southwestern Europe from Spain through Italy, a distribution consistent with natural spread from an origin in southern France.[13, 14]

Umina *et al.*[6] recently provided some evidence of sensitivity shifts to neonicotinoids in *M. persicae* populations within Australia. Using a leaf-dip bioassay technique, the authors investigated the response of 14 aphid populations to imidacloprid. Two populations (one from Queensland and one from Western Australia) showed higher survivability when compared with a known susceptible population. Although the methodology used was not ideal for testing neonicotinoid chemicals,[15] differences in the survivability between populations found by Umina *et al.*[6] were within the bounds of resistance ratios conferred by the overexpression of *CYP6CY3* as reported by Puinean *et al.*[10] In this study, we use a combination of bioassays and biomolecular assays to thoroughly explore the nature of neonicotinoid resistance in Australia.

2. METHODS

2.1 Aphid collections and culturing

Between July 2013 and April 2015, *M. persicae* populations were collected from agricultural regions of Queensland (QLD), New South Wales (NSW), Victoria (VIC), South Australia (SA), Tasmania (TAS), and Western Australia (WA). Populations

were collected from nine locations in eastern Australia (QLD, NSW, VIC, TAS, and SA) and two locations from WA. At each location, global positioning system coordinates and plant host were recorded. Aphids were collected from canola crops, vegetable crops, and host weeds (Table 1). Collection was targeted at paddocks with a relatively high pesticide load and intensive cropping history. Field populations were then isolated in the laboratory to ensure the absence of parasitoids, using the method described in Umina *et al.*,[6] and the unparasitised survivors used to found colonies used in this study. Aphids were reared asexually on radish plants (*Raphanus sativus* L.) in small tubs with gauze windows maintained at 24°C and a photoperiod of 16:8 h (L:D). A control susceptible aphid population originally collected from Shepparton (VIC), and maintained in the laboratory since 2002, was used as a direct comparison in all bioassays.

2.2 Topical bioassays

Each aphid population was screened against imidacloprid 200 g/L (Nuprid 200SC; NuFarm Laverton, VIC, Australia). Laboratory insecticide bioassays were undertaken to determine the extent of *M. persicae* resistance to imidacloprid using a topical application technique according to Puinean *et al.*[10] Using a fine paintbrush and avoiding first-instar nymphs, eight apterous aphids were placed on the abaxial surface of a Chinese cabbage leaf disc (*Brassica napus* L var *chinensis*) sitting on 1% (w:v) agar in a 35 mL petri dish. Aphids were allowed to settle for at least 3 h before being dosed individually. Imidacloprid concentrations were applied using a 0.01 µL - 0.5 µL syringe to deposit a 0.25 µL droplet directly onto the prothorax of each individual aphid. Six concentrations that resulted in application of 0.01 ng, 0.03 ng, 0.1 ng, 0.3 ng, 1 ng, and 10 ng of imidacloprid per aphid were tested. These concentrations are

equivalent to 0.0008, 0.0024, 0.008, 0.024, 0.08, and 0.8 times the recommended field rate. A stock solution was made by adding 1 mL of chemical to 99 mL of water and this was further diluted using acetone to create the appropriate concentrations. Control aphids were dosed with 0.25 µL of acetone only. Doses were applied to aphids underneath a microscope to ensure accurate placement of droplets. Petri dishes were then inverted to simulate feeding conditions in the field, and placed at 20°C with a photoperiod of 16:8 h (L:D). Five replicates were used for each concentration tested and the responses were assessed after 72 h. Aphids were scored as alive (vibrant and moving freely), dead (not moving over a 5 second period), or incapacitated (inhibited movement). As incapacitated aphids invariably die and therefore do not contribute to the next population, they were pooled with dead individuals for data analysis.

Aphid mortality data from the five replicate petri dishes per concentration was pooled for each population and subjected to probit regression analysis, using modified R script from Johnson *et al.*[16] Lines were fitted to dose-mortality data on a log-probit scale for each pesticide using 'glm' in the R statistical package.[17] From these lines the lethal dose 50% (LD_{50}) values and accompanying 95% confidence intervals were calculated using Fieller's method, with correction for heterogeneity where appropriate.[18] Results from each aphid population were then compared with results from the susceptible control population within each assay to investigate differences, and where appropriate calculate resistance ratios. We also examined the hypotheses of parallelism (slopes not significantly different) and equality (slopes and intercepts not significantly different) with likelihood ratio ratio tests. Analyses were conducted using R version 3.3.1.[14]

2.3 Biomolecular assays and genotyping

We screened three individual aphids from each field-collected population for two different resistance mechanisms to imidacloprid: the R81T mutation, and increased copy number of the *CYP6CY3* gene.[10, 19] Three individual aphids from the control susceptible population were also screened against these resistance mechanisms.

For the R81T mutation we followed the methods described Puinean *et al.*[19] using a TaqMAN single nucleotide polymorphism (SNP) assay (Life Technologies) to distinguish susceptible and resistant alleles. DNA was extracted from individual aphids using a QIAGEN DNeasy Blood & Tissue Kit (Qiagen) with the RNase A treatment step. DNA quality and quantity was assessed by Qubit Fluorometric Quantitation (Thermo Fisher Scientific) and by running an aliquot on a 1.5% (w:v) agarose gel. The DNA was then diluted to 2 ng/µl and 2 µl used in PCR. The TaqMAN SNP PCR assays were undertaken on a Roche LightCycler 480 system in a 384-well format. 10 µl reactions containing 5 µl of 2 × Kapa Probe Force Universal qPCR mix (Geneworks), 0.25 µl 40 × TaqMAN Gene Expression Assay, 2.75 µl ddH_2O and 2 µl of genomic DNA were prepared in triplicate. Included in each 384-well assay plate were control reactions containing DNA from aphids confirmed resistant and susceptible to the assayed SNP and controls with no DNA template. The amplification conditions were: 3 min at 98°C, followed by 10 s at 95°C and 20 s at 60°C for 40 cycles. The endpoint genotyping module of the LightCycler 480 software package was used to call SNP alleles. The performance of the assay and amplification of each SNP was confirmed from the control reactions.

We used quantitative PCR (qPCR) to determine *CYP6CY3* gene copy number relative to the three control genes *ace*, *para* and *actin* using the approach of Puinean *et al.*[10] The qPCR assays were undertaken on a Roche LightCycler 480 system in a 384-well format. 10 µl reactions containing 5 µl of 2 × Kapa Probe Force Universal qPCR mix (Geneworks), 1 µl LightCycler 480 High Resolution Melting Master (Roche), 0.25 µl of each primer (final concentration 0.25 µM), 1.5 µl ddH_2O and 2 µl of genomic DNA were prepared in triplicate. Included in each 384-well assay plate was a 5 point 10-fold dilution series of DNA to assess the efficiency of each primer and controls with no DNA template. The amplification conditions were: 3 min at 98°C, followed by 15 s at 95°C, 15 s at 57°C and 20 s at 72°C for 40 cycles with a final 72-95°C ramp (1°C every 5 s) to examine melting profiles. The relative quantification module of the LightCycler 480 software package was used to calculate the gene copy number relative to the three control genes.

We also undertook genetic analysis to identify the clonal makeup of each population, following the methods described in Umina *et al.*[6] Ten individual aphids from each field collected population were genotyped at 10 microsatellite loci: M35, M37, M40, M49, M55, M63, M86, myz2, myz9 and myz25.[20] These individuals were also screened across a number of resistance mechanisms, including the *E4/F4* esterase genes, *kdr*, super-*kdr*, and *MACE*. These assays followed the methods described by Anstead *et al.*[21, 22] Ten individual aphids from the control susceptible population were also screened against these resistance mechanisms.

3. RESULTS

Our findings demonstrate resistance to imidacloprid is present in Australian *M.*

persicae, and more widespread than expected. Of the 13 populations screened across Australia, six populations were found to have an elevated level of resistance to imidacloprid (Table 2). Populations demonstrated relatively low levels of resistance to imidacloprid when compared with the susceptible population. This is evident when comparing the percentage mortality of each population at the discriminating dose of 0.1 ng a.i. per aphid, which approximately represents the LD_{99} value for susceptible populations. Three resistant populations show mortality of 50% at this dose, and the other three populations display ≤82% mortality (Fig. 1). Resistance ratios for the six populations ranged between ~4.5-fold (Virginia) and ~25-fold (Airville) (Table 2). Populations with resistance were found in all states except TAS and VIC. The regression lines of all 13 field-collected populations had significantly different intercepts from the susceptible population, while three populations (Airville, Wesley Vale and Penfield) had significantly different slopes from the susceptible population (Supplementary Table 1).

Investigations into the resistance mechanism(s) of aphids confirmed our bioassay results. No individuals from the control susceptible population were found to have the R81T substitution in the ² 1 subunit of the nicotinic acetylcholine receptor, and exhibited no increased copy number of the *CYP6CY3* gene (Table 2). The mutation producing the R81T substitution was not detected in aphids showing phenotypic resistance, but in the same aphids there was an increase in copy number of *CYP6CY3*. Copy numbers ranged from 2.8-6.7 (Table 2). Even those aphid populations that did not show a significant difference in LD_{50} values from the control susceptible population had a small increase in *CYP6CY3* copy number.

Genotyping of aphids revealed three distinct multi-locus clones across all field collected populations (Table 2), with each population consisting of only a single multi-locus clone. *CYP6CY3* copy number was consistent within each clonal type, with all clone A populations having copy numbers of ~3, while clone C populations had copy numbers of ~7. The single clone B population (Penfield) had a copy number of 2.8. Aphids from all three clones carried resistant alleles for *MACE* and super-*kdr*, as well as amplification of the *E4* esterase gene. Conversely, every individual from the control susceptible population was found to carry susceptible alleles for *kdr*, super-*kdr* and *MACE*, and exhibited no amplification of either the *E4* or *FE4* esterase genes.

4. DISCUSSION AND CONCLUSIONS

For the first time, we have demonstrated resistance to neonicotinoids in *M. persicae* populations from Australia. Resistance ratios and dose-response curves indicate low resistance to neonicotinoids in almost half of the 13 populations tested, with the highest observed resistance ratio of ~25-fold observed in a population from QLD. These levels of neonicotinoid resistance in *M. persicae* populations are consistent with the metabolic resistance conferred by increased expression and/or copy number of the P450 gene, *CYP6CY3*. Our genetic screening indicates that this is very likely to be the principle mechanism for neonicotinoid resistance currently present in Australia.

Although neonicotinoid resistance is found in *M. persicae* across Europe and in the USA (ARPD (http://www.pesticideresistance.org/)), it appears to have arisen relatively recently within Australia. Of the two main resistance mechanisms to neonicotinoids found in *M. persicae* (target-site and metabolic), metabolic resistance

is more commonly found worldwide.[13, 14] With this confirmation of metabolic resistance in multiple *M. persicae* populations, Australia now joins four other continents (North America, Asia, Europe and Africa) that have populations of *M. persicae* with neonicotinoid resistance. Importantly, *M. persicae* populations in some parts of Australia are now resistant to four different IRAC mode of action groups: pyrethroids (3A), carbamates (1A), organophosphates (1B) and now, neonicotinoids (4A). Despite the current scale of resistance, insecticides remain a major component of many aphid control programs in Australia, and resistance management strategies, based on mode of action rotation, are of crucial importance in preventing resistance becoming widespread.

The resistance ratios displayed by the six resistant populations in our study are similar to those found by Umina *et al.*[6] Similarly low levels of resistance to neonicotinoids have been observed in many countries worldwide.[11, 23, 24] It has been suggested that this low-level resistance, conferred by the over-production of the *CYP6CY3* enzyme, is likely the consequence of enhanced detoxification, a byproduct of tolerance to nicotine.[11, 12] This is further confirmed by the fact that low-level metabolic resistance to neonicotinoids was reported in tandem with the first commercial releases of this insecticide group.[11, 25, 26] Tobacco has never been widely grown in Australia, and tobacco farming ceased in 2006.[27] Additionally, we detected imidacloprid resistance in isolated populations in WA, where tobacco has never been grown. Hence, pre-selection for imidacloprid resistance in Australian clones is likely to have occurred prior to their introduction to Australia.

The three clones we identified in this study are widely distributed across Australia,

and all three are found across a range of horticulture and broadacre crops. These clones dominate *M. persicae* populations in Australia, accounting for up to 80% of field-collected populations (A. van Rooyen, unpub. data). Although individual clones had a consistent copy number of the *CYP6CY3* gene, there was no strong relationship between phenotypic resistance ratios and copy number. This is similar to other studies of this relationship in *M. persicae,*[28] as well as for other species and P450 genes.[29, 30] As the level of P450 gene expression is not necessarily linked to the gene copy number,[28, 29] this variation is not unexpected. The role of P450 genes in neonicotinoid resistance is complex. In addition to variation in copy number, the quantity of gene expression may not be linearly related to the level of resistance.[30] *CYP6CY3* expression is one of a number of genetic mechanisms conferring neonicotinoid resistance, while reduced penetration through the cuticle is also thought to play a role.[7]

Our study has found no evidence that high-level neonicotinoid target site resistance is present in Australia. Target site resistance (conferred through the R81T mutation) has only been found in connected holocyclic populations in southern Europe, and is far less common than low-level neonicotinoid metabolic resistance.[11] Target site resistance is semi-recessive,[31] and less likely to evolve where *M. persicae* populations are predominantly anholocyclic (asexual), which is generally the case in Australia.[32]

In the wake of the widespread high-level resistance to pyrethroids and carbamates in aphid populations, the use of neonicotinoids to control *M. persicae* in Australia has rapidly increased in the last 5-10 years. A large number of broadacre and horticultural crops have neonicotinoid seed treatments or seedling drenches prior to planting, as

well as foliar sprays for pest control during crop development. Currently, a third of insecticides registered for *M. persicae* control in Australia fall under the IRAC mode of action classification Group 4 (neonicotinoids) (APVMA (https://portal.apvma.gov.au/pubcris/)). Imidacloprid is the active in the majority of these products (~90%). The remaining actives include thiacloprid, thiamethoxam, acetamiprid, clothianidin and sulfoxaflor (APVMA (https://portal.apvma.gov.au/pubcris/)). Cross-resistance between different IRAC Group 4 chemicals is acknowledged between the closely related compounds imidacloprid, thiacloprid, thiamethoxam, acetamiprid, clothianidin, dinotefuran and nitenpyram.[11, 33] Cross resistance has also been detected in *M. persicae* between imidacloprid and sulfoxaflor, however only when the neonicotinoid resistance mechanism is caused by the R81T target site mutation.[11]

Although enhanced *CYP6CY3* gene expression confers some level of resistance to neonicotinoids in topical bioassays, so far this enhanced expression has only impaired field efficacy. We are not aware of any complete field failures involving neonicotinoids when applied at full registered rates. Despite this, the extent of resistance observed in our study, coupled with the current reliance on neonicotinoids for control of *M. persicae* in a multitude of crops is concerning. It is well recognised that *M. persicae* can establish resistance to new chemistries quickly. It is imperative that resistance management strategies are employed to manage this pest, in particular only spraying insecticides when economically necessary, and incorporating nonchemical control methods whenever possible.

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Table 1. Site details for *Myzus persicae* populations collected across Australia between July 2013 and April 2015.

Population	State	Date collected	Host plant	Latitude	Longitude
Erigolia	NSW	11/08/2014	*Brassica napus*	-33.8491	146.4206
Ariah Park	NSW	11/08/2014	*Brassica napus*	-34.4026	147.2625
Karkoo	SA	22/07/2014	*Brassica napus*	-34.0295	135.7300
Penfield	SA	10/10/2014	*Solanum melongena*	-34.6954	138.6553
Virginia	SA	14/08/2014	*Capsicum annuum*	-34.6743	138.5561
Gumlu	QLD	06/08/2013	*Capsicum annuum*	-19.8647	147.6705
Airville	QLD	06/08/2013	*Capsicum annuum*	-19.6310	147.3460
Richmond	TAS	03/11/2014	*Brassica oleracea var. capitata*	-42.7333	147.4333
Table Cape	TAS	07/04/2015	*Raphanus raphanistrum*	-40.9498	145.7085
Wesley Vale	TAS	07/04/2015	*Brassica oleracea var. italica*	-41.1900	146.4550
Bindoon	WA	23/07/2013	*Brassica napus*	-31.3800	116.0970
Pemberton	WA	15/01/2015	*Raphanus raphanistrum*	-34.4400	116.0500
Werribee	VIC	09/12/2014	*Brassica oleracea var. capitata*	-37.9000	144.6640

Table 2. Summary of resistance responses for field-collected *Myzus persicae* populations against imidacloprid at 72 h computed from probit regression models.

Bioassay	Population	*n*	LC_{50} (95% CI) ng a.i. per aphid	Slope (± SE)	df	sig.	copy no.	R.R.	Clone
A	Susceptible	275	0.008 (0.00002-0.03)	0.98 (±0.29)	4	<0.0001	1.1	1	S
	Airville	280	0.209 (0.099-0.491)	1.56 (±0.27)	4	<0.0001	6.7	24.7*	C
	Pemberton	291	0.155 (0.039-0.633)	0.91 (±0.19)	4	<0.0001	2.81	18.3*	A
	Bindoon	290	0.189 (0.125-0.291)	1.03 (±0.12)	4	<0.0001	2.86	22.3*	A
B	Susceptible	260	0.009 (0.004-0.014)	1.78 (±0.37)	4	<0.0001	1.1	1	S
	Richmond	226	0.018 (0.003-0.039)	1.72 (±0.42)	4	<0.0001	3.18	2	A
	Gumlu	235	0.021 (0.011-0.033)	1.17 (±0.19)	4	<0.0001	3.42	2.3	A
	Table Cape	224	0.025 (0.002-0.069)	1.38 (±0.37)	4	<0.0001	3.23	2.8	A
C	Susceptible	265	0.007 (0.002-0.02)	1.15 (±0.23)	4	<0.0001	1.1	1	S
	Virginia	276	0.034 (0.024-0.051)	1.35 (±0.15)	4	<0.0001	6.02	4.3*	C
	Wesley Vale	265	0.011 (0.009-0.015)	2.80 (±0.37)	4	<0.0001	3.34	1.8	A
D	Susceptible	277	0.01 (0.002-0.059)	2.71 (±0.86)	4	<0.0001	1.1	1	S
	Penfield	272	0.004 (-)	1.59 (±0.62)	4	<0.0001	2.76	0.4	B
	Werribee	266	0.021 (0.006-0.113)	2.04 (±0.58)	4	<0.0001	3.2	2	A
E	Susceptible	270	0.005 (0.001-0.01)	1.61 (±0.4)	4	<0.0001	1.1	1	S

	Ariah Park	264	0.021 (0.008-0.037)	1.83 (±0.35)	4	<0.0001	2.95	3.9	A
	Karkoo	272	0.024 (0.017-0.033)	1.93 (±0.27)	4	<0.0001	3.05	4.5*	A
F	Susceptible	270	0.018 (0.013-0.023)	2.54 (±0.4)	4	<0.0001	1.1	1	S
	Erigolia	276	0.08 (0.049-0.133)	3.05 (±0.55)	4	<0.0001	2.82	4.5*	A

n = number of aphids tested, , CI = confidence intervals, SE = standard error, df = degrees of freedom, sig. = chi-squared goodness of fit test, copy no. = copy number of *CYP6CY3,* R.R. = resistance ratio. * indicates populations considered to be resistant.